Apparent mutagenicity of *N*-nitrosothiazolidine caused by a trace contaminant

Summary

The identification of *N*-nitrosothiazolidine (NTHZ) in smoked meat products prompted us to evaluate this compound for mutagenicity by the Salmonella assay. NTHZ was prepared in 99 + % purity by the nitrosation of the cysteamine-formaldehyde reaction mixture without isolation and purification of the resulting amine, and from thiazolidine, directly. Mutagenic activity was observed with TA100 without metabolic activation in the former, but not the latter preparation. An examination of the precursors, reaction intermediates, and HPLC separation of the NTHZ from the mutagenic product demonstrated that the genotoxic activity resulted from a synthesis-produced trace contaminant.

N-Nitroso compounds in foods are of importance because they may contribute to the total human mutational load and to the etiology of cancer (NAS, 1981). Therefore, we were concerned by the observation of an occasional unknown peak on the gas chromatograph–thermal energy analyzer (GC–TEA) chromatogram during the analysis of fried bacon for volatile nitrosamines. This component was later identified as *N*-nitrosothiazolidine (NTHZ) (Fig. 1) (Kimoto et al., 1982; Gray et al., 1982).

After development of a dual column chromatographic procedure to permit isolation of the compound (Pensabene and Fiddler, 1982) a limited survey of smoked meat products demonstrated that 1.7–19 ppb NTHZ was present in 23 of 70 samples, other than cure-pumped bacon (Pensabene and Fiddler, 1983a). These products included: Lebanon bologna (50% of samples), cure-pumped ham (40%), pepperoni (44%), Canadian bacon (60%), beef and pork breakfast strips (71%), and dry cured bacon (100%). In addition, 71% of the cure-pumped bacon samples contained NTHZ. Other experiments demonstrated that raw, cure-pumped bacon contained higher levels of NTHZ than either fried, baked, or broiled bacon (Pensabene and Fiddler, 1983b). The data also indicated that the nitrosamine was associated with the smoking step in smokehouse processing.

NTHZ was reported to be a direct-acting mutagen in *Salmonella typhimurium* TA100; mutagenic activity was suppressed by S9 rat-liver homogenate (Mihara and Shibamoto, 1980; Sekizawa

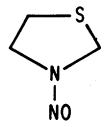

Fig. 1. Structure of *N*-nitrosothiazolidine.

and Shibamoto, 1980). The carcinogenic potential of NTHZ has not been reported. Since we were able to detect this nitrosamine in smoked cured meat products, and because of its reported bacterial mutagenicity, we performed studies to verify its genotoxic potential with Salmonella.

Experimental

Materials

Cysteamine hydrochloride, thiazolidine, formaldehyde (37%), sodium nitrite, and all other reagents were purchased from commercial suppliers and used without further purification.

N-Nitrosothiazolidine synthesis

(1) Thiazolidine: Sodium nitrite (0.30 mole) was added slowly to a cooled (0°C), stirred, aqueous solution of thiazolidine (0.28 mole) and 9 N sulfuric acid (0.28 mole). After addition, the cold mixture was stirred for an additional hour, then for 1 h at room temperature. The reaction mixture was extracted with dichloromethane (DCM); the DCM extract was washed with 30% KOH, dried over anhydrous sodium sulfate, and concentrated on a rotary evaporator. The oily residue was distilled in vacuo (85°C at 0.55 mm). (2) Cysteamine-formaldehyde: An aqueous solution of cysteamine hydrochloride (0.18 mole) and formaldehyde (0.18 mole) was stirred at room temperature for 24 h. This solution was cooled to 0°C then 9 N sulfuric acid (0.18 mole) and sodium nitrite (0.20 mole) were slowly added. The mixture was stirred at 0°C for 1 h and at room temperature for an additional hour. Isolation of NTHZ was as described by Ray (1978), except DCM was used to extract the reaction mixture. The rest of the procedure was as indicated above.

N-Nitrosothiazolidine confirmation

NTHZ from both sources was confirmed by GLC–MS (Hewlett Packard, Model 5992B *), with no apparent differences in their spectra when run under conditions described previously (Kimoto et al., 1982). No difference was observed in the spectra obtained from the NTHZs when scanned from 260 to 400 nm on a Hewlett-Packard Model 8450A UV/VIS spectrophotometer, using hexane as the solvent. Both samples of NTHZ were greater than 99% pure as indicated by GLC using a 1.8 m × 2 mm column containing 15% Carbowax 20M-TPA on 60/80 Gas Chrom P.

HPLC analysis

50 μl of NTHZ distillate obtained from cysteamine-formaldehyde-nitrite was diluted with 50 μl hexane–DCM (1 : 1) and separated by HPLC (Milton Roy) using a 3.2 mm × 25 cm 5 μm Spherisorb silica column and a variable wavelength detector (Perkin-Elmer, Model 55) set at 365 nm, the maximum absorbance of NTHZ. A linear solvent gradient of 100% hexane to 100% DCM over a 15-min interval was employed with a flow rate of 1 ml/min. Sample fractions were collected after 2 min for 4- or 6-min intervals up to 30 min.

Mutagenicity assay

Mutagenicity assays were performed in accordance with the preincubation method described by Kitamura et al. (1981). *Salmonella typhimurium* TA100 (a gift from Dr. B.N. Ames, Berkeley, CA) was used as the indicator organism (Maron and Ames, 1983). Approximately 2×10^8 cells were inoculated into sterile test tubes. To this was added 100 μl of dimethyl sulfoxide (DMSO), alone or with the dissolved test compound, and 500 μl sodium phosphate buffer (0.1 M, pH 7.4), or an Aroclor-induced rat-liver S9 mix consisting of 8 μM $MgCl_2$, 33 μM KCl, 5 μM glucose-6-phosphate, 4 μM NADP, and 0.1 M sodium phosphate (pH 7.4). The solutions were incubated for 20 min at 37°C. At termination, 2 ml of 50°C soft top agar (0.5%) was added to the incubating solutions. They were then mixed and poured onto petri dishes containing minimal medium, as described by Miller and Buchanan (1983). After an hour the dried plates were inverted and incubated for 48 h at 37°C. Plates were then checked for appearance of normal background growth. His^+ revertant colonies were counted using a Biotran automated colony counter (New Brunswick Scientific Company). All experiments were performed 2–3 times, using triplicate plates per determination. A posi-

tive mutagenic response was defined as a minimum of a doubling above the solvent reversion yield. Appropriate solvent and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) controls were included each day with the experimental samples. For experiments with S9, benzo[*a*]pyrene (BaP) was used as a positive control. Spontaneous revertant yield for TA100 was 89 ± 8 revertants; for 10 μg/plate MNNG and 10 μg/plate BaP, the yield was 2253 ± 174 and 452 ± 25 revertants, respectively. Genetic markers were checked routinely according to Maron and Ames (1983).

Results

NTHZ synthesized by the direct nitrosation of thiazolidine, which we used earlier to confirm the identity of the nitrosamine in bacon, was tested by the Ames mutagenicity assay procedure. This compound was not mutagenic to TA100 over a 2-log dose range. Similar results were obtained by the addition of rat-liver S9. Since these results were contrary to those reported by Mihara and Shibamoto (1980), we synthesized the NTHZ from cysteamine-formaldehyde-nitrite (their method) and found the product to be mutagenic (maximum response was approximately 9 × above spontaneous level in this experiment). Addition of S9 decreased the mutagenic response significantly thus supporting the observation of Mihara and Shibamoto (1980). There was no evidence of cytotoxicity at the concentrations used in these experiments. The mutagenic responses of NTHZ synthesized by the 2 different methods are shown in Fig. 2. Values are expressed as revertants/plate. It is likely that the other investigators did not detect mutagenicity induced by NTHZ, but by some other compound. Our results suggest that the mutagenic species of the cysteamine-formaldehyde-nitrite reaction, which we will henceforth designate 'NTHZ', was either a trace contaminant formed as a result of a side reaction or a residual reaction precursor.

For this reason, we tested individually formaldehyde, cysteamine, thiazolidine, and nitrite for potential genotoxic activity, using the same assay conditions as for the nitrosamine. The results are shown in Fig. 3. Formaldehyde was strongly mutagenic at the 5 μM level, but became cytotoxic at higher doses; Donovan et al. (1983) has made a similar observation. However, residual formaldehyde was probably not the mutagenic species since its presence in the purified NTHZ was unlikely because it is both volatile and highly reactive. Cysteamine was found not to be mutagenic over a 0–30 μM dose range. This compound was, in fact, shown to offer protection against

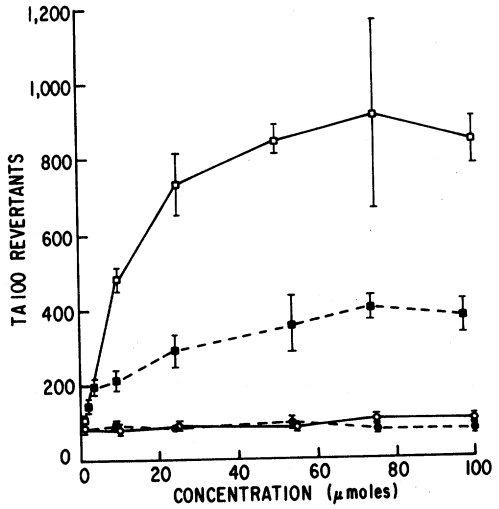

Fig. 2. Effects of 2 synthesis methods on the bacterial mutagenicity of NTHZ. Cysteamine-formaldehyde-nitrite reaction: □, −S9; ■, +S9; thiazolidine/nitrite: ○, −S9; ●, +S9.

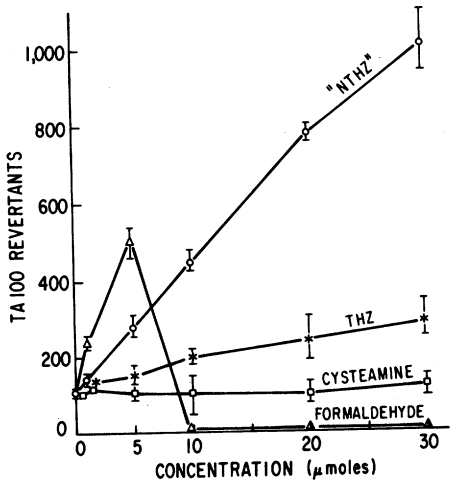

Fig. 3. Mutagenic effects of NTHZ and precursors toward Salmonella. ○, NTHZ; *, thiazolidine; □, cysteamine; △, formaldehyde.

genotoxic agents through what was thought to be radical scavenging activity (Bianchi et al., 1982). Thiazolidine was tested since mutagenic activity was reported by Mihara and Shibamoto (1980). We observed a doubling of TA100 revertants at 20 μM/plate, which was not sufficient to account for the mutagenicity of 'NTHZ'. The nitrite results, not shown on the figure, gave no evidence of mutagenic activity between 0 and 100 μM/plate under the conditions employed which involved the use of a pH 7.4 buffer. Under acidic conditions, nitrite, or more precisely nitrous acid, would be expected to be mutagenic, since it is known to deaminate nucleotides (Nicholson-Guthrie, 1970). Generally, these results suggested that the genotoxic response in NTHZ was not entirely due to residual reaction mixture precursors.

The purification steps of 'NTHZ' synthesis were next investigated individually for mutagenic activity (Fig. 4). The reaction mixture of cysteamine-formaldehyde-nitrite before distillation showed high levels of mutagenic activity directly with TA100. The maximum response was observed at 1

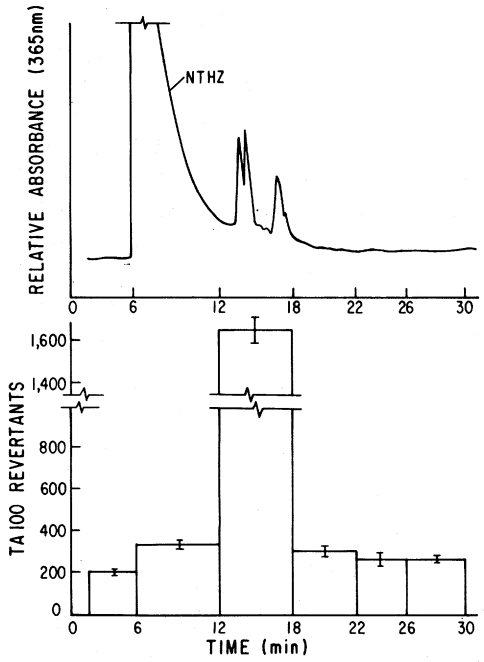

Fig. 5. HPLC separation of NTHZ from observed Salmonella mutagenic activity.

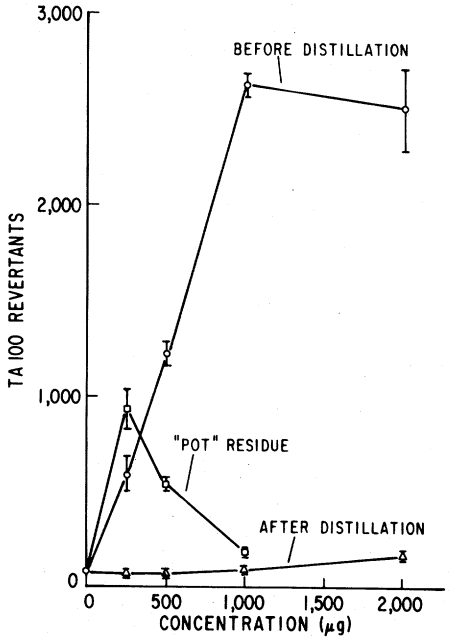

Fig. 4. Mutagenic activity during NTHZ purification. ○, reaction before distillation; △, final distillate; □, reaction mixture residue.

mg/plate. At higher concentrations cytotoxicity was evident, as indicated by reduced mutant yields and sparse bacterial lawns. The first distillate collected (not present in the figure) showed no mutagenic activity, and, in this sample, the final distillate showed only weak mutagenic activity. The residue after distillation demonstrated the steepest mutagenic slope; however, cytotoxic effects offset mutagenic responses above 250 μg/plate. Thus, results from the experiment shown in Fig. 4 indicate that the mutagen generated during the cysteamine-formaldehyde-nitrite reaction is a high-boiling compound.

HPLC studies were then carried out using a silica column to further characterize the mutagenic contaminant. The chromatogram is shown in Fig. 5. The fraction collected between 6 and 12 min, independently determined to contain NTHZ, exhibited no mutagenic activity. Whereas, the fraction between 12 and 18 min, that contained several small peaks, showed strong mutagenic potential as indicated in the same figure. The other fractions were found to not exhibit mutagenic activity.

Discussion

The present study provides evidence showing that NTHZ is not a direct-acting mutagen as detected by Salmonella TA100, but rather detectable genotoxic activity was dependent upon the method of synthesis. No mutagenic activity was observed in samples synthesized by the direct nitrosation of thiazolidine in the presence or absence of rat-liver S9. On the other hand, the cysteamine-formaldehyde-nitrite reaction product exhibited strong direct-acting mutagenic activity in Salmonella TA100 that could be reduced with rat-liver S9, as reported previously (Mihara and Shibamoto, 1980; Sekizawa and Shibamoto, 1980). We were able to separate by HPLC the mutagenic component from the true NTHZ, although efforts to repeat the separation for characterization and identification have failed because the active component appears to be labile, especially when isolated. Therefore, we concluded that the mutagenic activity observed with NTHZ was due to 1 or more contaminants resulting from the cysteamine-formaldehyde-nitrite reaction that was not present when the nitrosamine was synthesized from thiazolidine and nitrite. It should be noted that the contaminant(s) is probably present in very low concentration, because of the identical MS and UV spectra, and GC chromatograms of the NTHZ synthesized by the 2 different methods. Therefore, given the relatively strong mutagenic response, the active contaminant(s) would have to be considered a potent direct-acting mutagen.

The data presented in this study suggest that cysteamine-formaldehyde-nitrite side reactions are important determinants for mutagen formation. This is of concern because the reactions that form NTHZ or closely related compounds, even if not mutagenic themselves, may artifactually generate mutagenic components. Two natural situations have been shown to produce NTHZ. First, previous work from this laboratory demonstrated that NTHZ is found in cured meat products and is associated with smokehouse processing (Pensabene and Fiddler, 1983b). Secondly, similar compounds have been reported to be formed in vivo. Tsuda et al. (1983) and Ohshima et al. (1983) recently reported that *N*-nitroso-L-thioproline and 2-methylthioproline are present in human urine. These compounds can be decarboxylated to form NTHZ and 2-methyl-NTHZ, respectively. Both groups of investigators suggested that these products are formed from the reaction of cysteine, and formaldehyde or acetylaldehyde. In both instances natural condensation products from aldehyde-thiol-nitrite were observed. Therefore, it is reasonable to suggest that similar mutagens may be formed in these systems as in the cysteamine-formaldehyde-nitrite model system.

The mutagenicity of sulfur and nitrogen heterocyclic compounds is not without precedent. Voogd et al. (1983) studied 27 amino and nitro heterocyclic sulfur compounds used as veterinary and medical drugs. All sulfur compounds with nitro functions and some aminothiazoles were direct-acting bacterial mutagens when tested with base-pair substitution tester strains. Furthermore, some heterocyclic nitrosamines have been shown to be mutagenic. For example, *N*-nitrosothiomorpholine was mutagenic toward Salmonella TA1535 in the presence of mouse-liver S9 (Zeiger and Sheldon, 1978) and was carcinogenic (Garcia et al., 1970). Nonetheless, the Salmonella data reported in the present study does not indicate a positive mutagenic response for the 5-member ring homolog.

In conclusion, the results of this study indicate NTHZ is not mutagenic in our test systems; rather, a mutagenic contaminant was formed during the synthesis of NTHZ by the cysteamine-formaldehyde-nitrite reaction. This may be of concern if the contaminant is shown to be formed in vivo or during the smoking of meat products. Efforts to determine the mechanism of formation are now in progress.

Note: Nitrosamines are potential carcinogens. Exercise care in handling these materials.

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